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Methicillin-resistant *Staphylococcus epidermidis* carrying biofilm formation genes: detection of clinical isolates by multiplex PCR

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Summary. *Staphylococcus epidermidis* is the most prevalent coagulase-negative *Staphylococcus* (CNS) and is a major cause of hospital bacteremia. Based on 18 reference strains and 149 *Staphylococcus* clinical strains, used in a novel multiplex PCR method, the aim of this study was to identify *S. epidermidis* with respect to the sequence of three genes: *recN*, which encodes a recombination/repair protein, *mecA* (methicillin resistance), and *icaAB*, which is involved in biofilm formation. Amplicons of 219 bp (*S. epidermidis-recN* gene), 154 bp (*mecA* gene), and 546 bp (*icaAB* genes) were obtained. Reliable results were achieved for 100% of the evaluated strains, suggesting that this new multiplex-PCR approach could be useful for the accurate identification of methicillin-resistant *S. epidermidis* with the potential to produce biofilm. [**Int Microbiol** 2011; 14(1):13-17]

Keywords: *Staphylococcus epidermidis* · molecular identification · methicillin resistance gene · biofilm formation genes · multiplex PCR

Introduction

Staphylococcus epidermidis is the most frequent coagulase-negative *Staphylococcus* (CNS) isolated from bloodstream infections [18]. Its prevalence is associated with its tendency to colonize central venous catheters and other implanted medical devices [21], which relies on its ability to develop a highly consolidated structure: the biofilm [9]. In particular biofilm-forming strains of *S. epidermidis* that are also methicillin-resistant (MRSE) have become a very serious clinical problem, as these infections are especially difficult to eradicate from the colonized devices [7].

To shed light on the clinical significance of *S. epidermidis* in infections and to provide data for control and epidemiological measures, strains of this organism must be reliably identified. However, the diagnosis of *S. epidermidis* currently depends on time-consuming conventional microbiological biochemical tests, which provide species identification and susceptibility testing albeit with low accuracy

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[6,17]. Thus, the development of accurate and simple methods to identify isolates of *S. epidermidis* and determine their methicillin resistance is important to establish the clinical relevance of *S. epidermidis* [16]. Moreover, simultaneous detection of the biofilm *icaAB* genes in clinical isolates may anticipate the possibility of biofilm colonization of catheters before it actually occurs, perhaps preventing the potentially fatal consequences for infected patients [14]. In the present study, we developed a multiplex polymerase chain reaction (PCR) based on the sequence of the *recN* gene, which encodes a recombination/repair protein [23], to identify *S. epidermidis* species. This novel technique also allows the simultaneous detection of genes that encode methicillin resistance and biofilm formation.

Materials and methods

The amplification conditions were established by using 18 bacterial strains that included reference strains of *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and other species of CNS (Table 1). The multiplex PCR was validated with 149 clinically relevant strains isolated from blood (64%), catheter tip (9%), surgical site (6%), prosthesis (4%), urine (3%), ocular secretion (3%) and other sites (11%) in samples obtained between 1994 and 2008 from patients in eleven Brazilian hospitals. The following species of *Staphylococcus* were evaluated: *S. epidermidis* (82 strains), *S. haemolyticus* (22), *S. hominis* (16), *S. aureus* (15), *S. cohnii* (4), *S. lugdunensis* (3), *S. warneri* (3), *S. capitis* (2) and *S. saprophyticus* (2). All 167 references and clinical strains were phenotypically characterized to the species-level according to Iorio and coworkers [10]. Ten tests were used: coagulase, hemolysis, clumping factor, pyrrolidonyl arylamidase, urease, alkaline phosphatase, susceptibility to novobiocin and desferrioxamine, and acid production from: D-trehalose and D-mannose. Moreover, 35 (23%) of the clinical strains, including 15 *S. epidermidis*, eight *S. haemolyticus*, eight *S. hominis*, two *S. cohnii*, and two *S. lugdunensis* were also characterized by PCR-RFLP [2] of the *groEL* gene, in accordance with the results of phenotypic identification. The strains were tested for methicillin susceptibility by the disk-diffusion method [4] using 30-μg cefoxitin disks (Oxoid, Hampshire, England). For PCR, the strains were initially grown on blood agar (Plast Labor, Rio de Janeiro, Brazil). Rapid DNA extraction was achieved by suspending five or six bacterial colonies in 150 μl of TE (10 mM Tris, 1 mM EDTA, pH 7.8) buffer and heating to 100°C for 10 min. After centrifugation at 20,000 ×*g* for 30 s, the supernatant was collected for the PCR [20].

The sequence of the *recN* gene, which was used to design the primers, was obtained from GenBank sequence database (accession no: CP000029). The primers were designed using the Oligo Explorer program [http://www.genelink.com/tools/gl-oe.asp]. Primer specificity was tested against the sequences in BLAST searches [http://www.ncbi.nlm.nih.gov/blast]. The oligonucleotide primers were purchased from Bioneer Oligo Synthesis Report (Daedeok-gu, Republic Korea). Primers designed in this study SepF (5′-CAG TTA ATC GGT ATG AGA GC-3′) and SepR (5′-CTG TAG AGT GAC AGT TTG GT-3′) were used to detect a 219-bp *recN* fragment (nucleotides 1330–1548). The primers MRS_1 (5′-TAG AAA TGA CTG AAC GTC CG-3′) and MRS_2 (5′-TTG CGA TCA ATG TTA CCG TAG-3′) [5] were used to detect a 154-bp fragment of *mecA* (methicillin-resistance), and the primers icaAB-F (5′-TTA TCA ATG CCG CAG TTG TC-3′) and icaAB-R (5′-GTT TAA CGC GAG TGC GCT AT-3′) to detect a 546-bp of *icaAB* genes [8]. The amplification was performed on a Thermal Cycler (Mastercycler, Eppendorf, Hamburg, Germany) using 25 μl of PCR mixture containing 3 μl of boiled cell lysate [20], 250 μM of each desoxynucleotide triphosphate (Life Technologies, California, USA), 1.5 U of *Taq* DNA polymerase (Biotools, Madrid, Spain), buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl and 3 mM $MgCl_2$, Biotools), 1 μM of the primers MRS1, MRS2; 0.4 μM of SepF and SepR; and 0.8 μM of icaAB-R, icaAB-F. Amplification conditions were: denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1min, with final extension at 72°C for 5 min. Amplified products were analyzed by 1.5% of agarose gel electrophoresis with GelRed 1X (Biotium, California, USA) and visualized and captured on UV transilluminator (Mini BIS Pro, DNR Bio Imaging Systems, Jerusalem, Israel).

Results and Discussion

The clinical strains were identified in species following conventional methods (Table 1). Results from the amplification of the *recN* gene, specific for *S. epidermidis* species, showed that all 82 (100%) *S. epidermidis* strains were detected (Table 1). Only strains identified as *S. epidermidis* by the phenotypic method were positive for *recN*. Among the 126 (85%) strains harboring the *mecA* gene and determined to be methicillin-resistant by the cefoxitin disk diffusion test, 78 (62%) were detected as MRSE. The results of the disk diffusion test were also in accordance with the PCR results for all *Staphylococcus* strains evaluated. Fifty-seven (70%) *S. epidermidis* strains harbored the *icaAB* genes, with the coexistence of *mecA* and *icaAB* genes observed in 55 (67%) of them. Figure 1 shows an agarose gel of amplified DNA corresponding to the *S. epidermidis* species-specific *recN* (219 bp), *mecA* (154 bp), and *icaAB* (546 bp) genomic segments detected by PCR multiplex. A single PCR of each gene was performed to confirm the negative results. The multiplex PCR showed total accordance with the single PCR test, with 100% specificity and sensitivity.

S. epidermidis is a commensal inhabitant of human skin and mucosa that may cause bloodstream infections [22]. Its pathogenicity in part relies on the presence or absence of *ica* and/or *mecA* genes, which are more frequently present in sepsis-causing strains [8,11]. Thus, the initial detection of *S. epidermidis* species might allow, in a second step, the determination of its clinical relevance based on the presence of *ica* and/or *mecA* genes.

The reference method for *Staphylococcus* identification, composed of 36 tests [1], is reliable but relatively cumbersome for use in routine laboratories. Thus, *S. epidermidis* continues to be reported as CNS [19]. Moreover, the detection of methicillin resistance by conventional tests is based

Table 1. Results obtained by *recN-mecA-icaAB* multiplex PCR method for identification of methicillin-resistant *Staphylococcus epidermidis* strains that carry biofilm formation genes

	Multiplex PCR			
Staphylococcus species	*S. epidermidis recN* gene	*mecA* gene	*icaAB* genes	Cefoxitin disk-diffusion[a]
Reference strains				
S. epidermidis ATCC 14990[1]	+	–	–	S
S. epidermidis ATCC 12228	+	–	–	S
S. epidermidis ATCC 35984[2]	+	+	+	R
S. haemolyticus ATCC 29970[1]	–	–	–	S
S. hominis ATCC 27844[1]	–	–	–	S
S. aureus ATCC 12600[1]	–	–	–	S
S. aureus ATCC 25923[3]	–	–	–	S
S. aureus ATCC 29213[3]	–	–	–	S
S. aureus ATCC 33591[4]	–	+	–	R
S. cohnii ATCC 29974[1]	–	–	–	S
S. lugdunensis DSMZ 4804[1]	–	–	–	S
S. warneri ATCC 10209	–	–	–	S
S. capitis ATCC 27840[1]	–	–	–	S
S. saprophyticus ATCC 15305[1]	–	–	–	S
S. xylosus ATCC 29971[1]	–	–	–	S
S. schleiferi DSMZ 4807[1]	–	–	–	S
S. simulans ATCC 27851	–	–	–	S
S. intermedius ATCC 29663[1]	–	–	–	S
Clinical strains identified by conventional method (no. of strains)				
S. epidermidis (55)	+	+	+	R
S. epidermidis (23)	+	+	–	R
S. epidermidis (2)	+	–	–	S
S. epidermidis (2)	+	–	+	S
S. haemolyticus (22)	–	+	–	R
S. hominis (8)	–	–	–	S
S. hominis (8)	–	+	–	R
S. aureus (13)	–	+	–	R
S. aureus (2)	–	–	–	S
S. cohnii (3)	–	–	–	S
S. cohnii (1)	–	+	–	R
S. lugdunensis (3)	–	–	–	S
S. warneri (2)	–	–	–	S
S. warneri (1)	–	+	–	R
S. capitis (2)	–	+	–	R
S. saprophyticus (1)	–	–	–	S
S. saprophyticus (1)	–	+	–	R

+ Presence or – absence of gene; R, resistant; S, susceptible; ATCC, American Type Culture Collection; DSMZ, German Collection of Microorganisms and Cell Cultures; [1]Type strain. [2]Biofilm producer control strain. [3]Susceptibility test control strain. [4]MRSA control strain.
[a]Disk with 30 µg of cefoxitin.

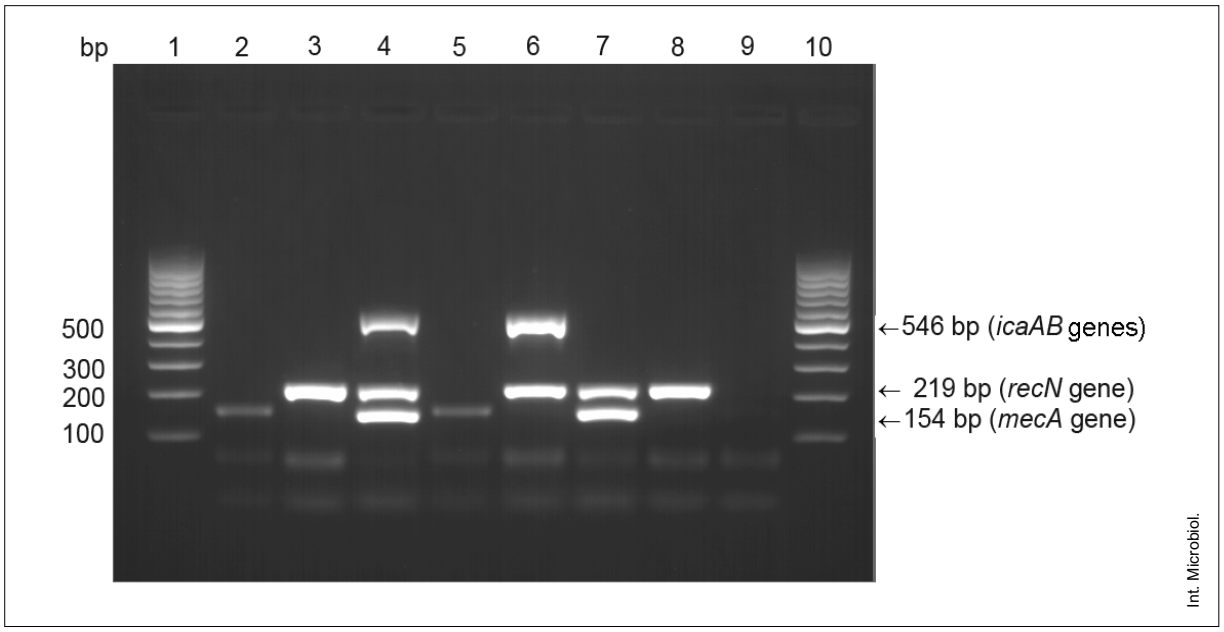

Fig. 1. Gel electrophoresis of multiplex PCR showing fragments of the *icaAB* (biofilm formation) genes (546 bp), the *Staphylococcus epidermidis* species-specific *recN* gene (219 bp), and the methicillin-resistance *mecA* gene (154 bp). Lanes 1 and 10, molecular size marker (100 bp ladder); lane 2, control strain of methicillin-resistant *S. aureus* (MRSA) ATCC 33591; lane 3, type strain of methicillin-susceptible *S. epidermidis* (MSSE) ATCC 14990; lane 4, control strain of biofilm producer and methicillin-resistant *S. epidermidis* (MRSE) ATCC 35984; lane 5, MRSA clinical strain; lane 6, clinical strain of biofilm producer MSSE; lane 7, MRSE clinical strain; lane 8, MSSE clinical strain; lane 9, reaction negative control.

on phenotypic expression, which can be heterogeneous [3]. Phenotypic methods, such as microtiter plate adherence and Congo red agar cultivation, have been used to evaluate biofilm production by staphylococci, but the results have been discordant, even when *ica* genes are present; that is, in strains with the potential to produce biofilm [11,12]. Thus, characteristics related to *S. epidermidis* species can be more reliably identified using molecular methods [6,13–15].

A PCR method to identify *S. epidermidis* species was previously reported [13,15]. However, to our knowledge, ours is the first report in which multiplex PCR was used to detect a single specific-species segment of *S. epidermidis* associated with segments of the *mecA* and i*caAB* genes. In this study, the sequence of the *recN* gene was used to design primers for the detection of *S. epidermidis* strains. *recN* encodes a recombination and repair protein that is found in bacterial genomes and can be used to predict whole-genome relatedness with high accuracy [23].

This study validated a PCR multiplex approach to detect MRSE strains also carrying biofilm formation genes, using 167 clinical and reference strains of different staphylococcal species. It showed 100% accuracy in the simultaneous detection of *recN*, *mecA*, and *icaAB* genes in all *S. epidermidis* strains evaluated. The method described herein is sensitive and specific, fast and feasible, and thus provides a new tool for the accurate identification of methicillin-resistant *S. epidermidis* with the potential to produce biofilms.

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Competing interests. None declared.

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